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Bacterial Leafspots of Pennsylvania Tobacco

I. Occurrence and nature of the microorganism associated with wildfire

Tobacco seedlings in Lancaster county

The Pennsylvania State College
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BACTERIAL LEAFSPOTS OF PENNSYLVANIA TOBACCO*

I. Occurrence and nature of the microorganism associated with wildfire

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THE CONSTANT THREAT of serious loss from leafspot diseases has confronted Pennsylvania tobacco growers for a number of years, and continues to be regarded today as the major hazard of the industry.

Following serious outbreaks in 1917 it was found *(23 and 24)* that the more important of these diseases, "wildfire", was associated with the activities of *Bacterium tabacum.* A second leafspot disease of tobacco was reported *(7 and 8)* which was named "angular leafspot" or "blackfire", in the same year. The organism associated with this disease was called *Bacterium angulatum.* The influence of climate, plant nutrition, and agronomic practices on the severity of these leafspot diseases was noted by these early investigators. Both groups of workers *(8 and 23),* however, concluded that these diseases had their origin in the seedbed and were carried to the field on the transplants.

For this reason all attempts at controlling the leafspot diseases of tobacco in the past two decades have been primarily attempts to eliminate from the seedbed the bacteria associated with the disease. The practices recommended have been termed "measures of sanitation" and have included removal of the seedbed from the vicinity of buildings, the steaming of soil, the disinfection of seed and implements, and the use of fungicides such as bordeaux mixture on both soil and plant. In certain districts producing a low nitrogen leaf tobacco, recommendations *(5, 18 and 19)* with respect to the nutrition of the plant have contributed to the control of disease.

The leafspot problem in the Lancaster area today continues to be serious. Sanitation apparently has failed to fully control the trouble, and the nutritional recommendations applicable in certain other districts are thought to be of little or no value in the production of cigar leaf and other high nitrogen tobaccos. This bulletin deals with the nature and identity of the microorganisms associated with leafspot diseases of tobacco, the influence of soil steaming on these microorganisms, and the normal bacterial flora of tobacco and other plants.

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BACTERIOLOGICAL METHODS

1. Analysis of soil samples.—Bacteriological examination of soil samples was made in the following manner: Soils were plated on sodium caseinate (nutrose) agar to determine total bacterial numbers. Fungi were determined by means of plate counts on glucose-peptone agar adjusted to pH 4.5. The numbers of green fluorescent bacteria were determined by the inoculation of asparagin-mineral salts broth to which crystal violet had been added to inhibit the growth of spore formers. The broth was inoculated with soil dilutions of 1 to 10, 1 to 50, 1 to 100, and 1 to 200, and the highest dilution in which growth of green fluorescent organisms occurred was used in calculating the minimum number of such organisms present in the sample. All incubations were carried out at 28° Centigrade.

The agglutination test was employed in the examination of green fluorescent bacteria for the presence of wildfire and angular leafspot organisms. Asparagin-mineral salts-crystal violet broth which showed growth of green fluorescent organisms was streaked on agar plates, colonies were picked and transferred to agar slants, and by means of the standard agglutination technique these subcultures were checked for evidence of agglutination in the presence of specific immune sera. Immune sera were prepared by the immunization of rabbits with known strains of the wildfire and angular leafspot organisms which had been obtained from other investigators. Agglutination titres of less than 1 to 300 were not considered specific. At frequent intervals strains identified by means of the agglutination technique were tested on a susceptible host as a further check on the identity of the organism.

2. Analyses of plant material.—Plant material was plated on nutrient agar plates for total numbers of bacteria, and from these plates green fluorescent colonies were picked and made the subject of special study. In those instances in which colonies of green-fluorescent organisms were not observed on nutrient agar the use of asparagin-mineral salts broth was resorted to. This substrate was used in the manner described in the discussion of soil analyses.

3. Cultures employed.—The bacterial cultures employed throughout this study were maintained on nutrient agar slants, pH 7.2, with frequent transfer and stored at 4° Centigrade.

4. Morphological and physiological characteristics.—The numerous morphological, cultural, and biochemical characteristics were determined in the usual manner with minor modifications which are noted in the thesis of one of the authors *(21)*.

5. Single-cell isolations.—Most of the cultures used as antigens in this study were the progeny of single cells obtained by the use of the Chambers micromanipulator. The Chambers technique as modified by Wright and McCoy *(25)* and Wright and Nakajima *(26)* with a few minor changes was used in this study. Cultures in the logarithmic growth phase (4 to 8 hours old) were taken up with a sterile micropipette and a series of droplets made on the underside of a sterile coverslip suspended above a moist chamber. The droplets were immediately examined microscopically and all droplets containing one cell were carefully charted. Incubation was carried out on the coverslip above the moist chamber and transfers were made to larger quantities of substrate after reproduction of the cells had occurred.

6. Serological techniques.—

A. PREPARATION OF ANTISERA:

Antigens for animal immunization were prepared by growing cultures in flasks with large agar surfaces. The flasks were inoculated with one-half ml of 18-hour broth cultures and incubated at room temperature for a period of 18 to 24 hours. The growth was checked for purity and then was washed off the agar with saline solution (0.85 per cent NaCl) containing 0.3 per cent tricresol (C.P.). The cells were removed from saline solution by centrifugation, were resuspended in saline solution, and again removed by centrifugation. Following this washing technique the cells were suspended in sufficient saline solution to obtain a density comparable to a No. 4 McFarland nephelometer standard, and permitted to remain for a period of 48 hours at 4° C.

Rabbits selected for immunization weighed not less than 2 kilograms each and were rejected in those cases in which the normal serum showed agglutinins at a dilution of 1 to 20. The animals received the antigens by intravenous injection. The initial dose was 0.25 ml The dosage was increased daily by 0.25 ml until the maximum dose of 2 ml was reached. The maximum dose was given daily until the tenth day. Following this series of ten injections of killed antigen, a series of injections of living antigen was administered. The initial dose of the living antigen was 0.25 ml. This was increased by 0.25 ml daily until a total of 6 ml of the living antigen had been administered. Following a 4-day rest period a trial bleeding was made. If a satisfactory titre had been reached, 30 to 40 ml of blood were removed aseptically from the heart, and allowed to clot; the serum was removed and stored under sterile conditons at 4°C.

B. AGGLUTINATION TECHNIQUE:

Sera dilutions from 1-20 to 1-10,240 were made in 1 ml portions of 0.85 per cent saline solution containing 0.3 per cent tricresol. One ml of the antigen suspension was added to each tube, giving final sera dilutions of from 1-40 to 1-20,480. The antigens were prepared from 18 to 24-hour cultures grown on nutrient agar which was removed and washed as described under the preparation of antisera and adjusted to a density comparable to a 1.5 to 2 McFarland nephelometer standard. Sensitization was carried out at 37°C. for a period of 24 hours followed by 12 hours at 4°C. and the degree of macroscopic agglutination recorded.

C. AGGLUTININ ABSORPTION:

The technique as outlined by Krumwiede, Cooper, and Provost *(16)* was followed. Large quantities of cells were grown at room temperature on nutrient agar for 25 hours, washed off with saline solution, filtered through cotton, and the cells recovered by centrifugation. The cells were again washed with saline solution and recovered by centrifugation. To a measured volume of the washed cells, serum and diluent were added according to the following formula:

$$(\text{MASS OF CELLS} - 10 \text{ PER CENT}) \times \text{DOSE FACTOR} = \text{TOTAL VOLUME}$$

$$\frac{(\text{MASS} - 10 \text{ PER CENT}) \times (\text{DOSE FACTOR} - 1)}{\text{SERUM DILUTION FACTOR}} = \text{SERUM REQUIRED}$$

$$\text{TOTAL VOLUME} - (\text{MASS OF CELLS} + \text{SERUM}) = \text{VOLUME OF DILUENT}$$

Trial absorption titrations were performed on each serum to determine optimal dose, serum dilution, and number of absorptions. A practice of using two absorbing doses was adopted as it resulted in a saving of cells and yielded more complete and uniform absorption. To avoid difficulty with differences in absorptive abilities of herterologous cultures, the absorbing dose was twice that necessary to completely absorb the homologous antiserum. As the absorptive capacity of cells in the "S" phase was found in this study to be less than that of cells in the "M" phase, three absorptions were made when absorbing with "S" cells.

The serum-bacteria suspensions were incubated at 37°C. for 3 hours with shaking every 30 minutes and were then kept overnight at 4°C. The cells were then removed by centrifugation, and to the supernatant serum another calculated volume of washed cells was added and the absorption again carried out. The cells were again removed by centrifugation, and in the case of absorption with "M" phase cells the serum was tested for degree of absorption by the standard macroscopic agglutination test previously described. In the case of absorption by "S" cells another calculated

volume of washed cells was added and absorption carried out for the third time before making the agglutination test. As a control, identical serum dilutions to which no absorbing cells were added were carried under the same conditions and finally tested by the standard agglutination technique.

7. Production of Dawson "S" forms.—The cultures in the "M" phase were grown in nutrient broth containing 10 per cent of the homologous "M" antiserum. Transfers were made daily and for the purpose of colony examination nutrient agar streak plates were also made daily from the broth containing the antiserum. In most cases examination of the streak plates showed that "S" forms appeared in abundance following the third or fourth serial transfer in immune serum broth. In the case of some cultures, however, prolonged serial transfer in the immune serum broth proved necessary.

Isolations were made from typical "S" colonies and antigens prepared for test against the homologous "M" phase antiserum. Failure to show agglutination at a titre of 1-320 was used as an arbitrary criterion of the removal of the capsule, at which time the culture was considered as representative of the "S" phase. Preparation of antisera in the case of the "S" phase antigens was carried out in the manner described in the case of "M" phase antigens.

EXPERIMENTAL RESULTS SHOWING NATURE OF ORGANISM

Morphological and physiological characteristics of green fluorescent isolates.—In the course of these studies of the microflora of soils and plants, isolations of more than 4000 strains of gram-negative fluorescent bacteria have been made in the bacteriological laboratories of The Pennsylvania State College. In addition approximately 100 cultures of *Pseudomonas* and *Phytomonas* were obtained from other laboratories and used for purposes of comparison. From this large number, 640 representative cultures were selected for study in this investigation. The morphological and biochemical characteristics of this group of bacteria were determined by employing the following tests: Staining for flagella, Gram reaction, hemolytic power, nitrate reduction, gelatin liquefaction, action in litmus milk, fermentation of glucose, of sucrose and of lactose, indol formation, hydrogen sulfide production, growth on potato, growth at different temperatures, and pigment formation.

These 640 green fluorescent bacteria were divided into two groups on the basis of the characteristics shown in table 1.

Table 1 shows that a small number of the 640 cultures were monotrichous, possessing only one polar flagellum, while 613 cul-

Table 1.—Classification of 640 fluorescent bacteria studied.

	MONO-TRICHOUS	LOPHO-TRICHOUS
Number of cultures	27	613
Per cent showing hemolytic activity	100.0	0.0
Per cent producing pyocyanin	63.0	0.0
Per cent showing reduction of nitrates	100	100.0
Per cent exhibiting growth at 37°C.	100	1.6
Per cent showing gelation liquefaction	100	98.4

tures showed 2 to 5 polar flagella or were lophotrichous. The type of flagellation was found to be unchanged by cultivation on the various media used in this study, and, although it was possible to effect the temporary removal of flagella by growing in broth containing phenol in appropriate concentrations, transfers from such broth resulted in a return to the original type of flagellation. All monotrichous strains were found to be actively hemolytic while none of the 613 lophotrichous forms were capable of lysing bovine or sheep red blood cells. Pyocyanin formation was shown only with certain of the 27 montrichous forms and only a small percentage of the lophotrichous types which did not liquefy gelatin were able to grow at 37°C. Nitrate reduction contrary to the findings of some workers *(9 and 24)* was found to occur with all of the 640 cultures.

On the basis of these as well as additional morphological and cultural characteristics, it was evident that the 27 monotrichous cultures conform to the description of *Bacterium pyocyaneum* Lehmann and Neumann *(17)*. This group of 27 strains also conforms to the description of *Pseudomonas aeruginosa* as given by Bergey et al. *(1)* with the exception of flagellation. Inasmuch as a number of the strains in this group were received from other laboratories as *Pseudomonas aeruginosa* and had originally been isolated from human lesions, the entire group of 27 has been considered representative of that species. Subsequently, Harris *(12)* found all members of this group to be serologically homologous in the decapsulated state and antigenically different from decapsulated representatives of the 613 lophotrichous strains.

Additional morphological and biochemical characteristics of the 613 lophotrichous forms permits their separation into 2 groups as shown in table 2.

It may be noted in table 2 that the 613 lophotrichous organisms were separated into 2 groups on the basis of their action in gelatin, litmus milk, and the ability to grow at 37°C. Group 1 comprising

Table 2.—The morphological and biochemical characteristics of 613 lophotrichous bacteria.

	GROUP 1	GROUP 2
Number of cultures	603	10
Polar flagella	2 to 5	2 to 5
Gram reaction	Negative	Negative
Shape	Short rods	Short rods
Growth at 37°C.	Little or no growth	Good growth
Agar slant	Filiform, greyish white	Filiform, greyish white
Gelatin stab	Crateriform to infundibuliform liquefaction	No liquefaction
Nutrient broth	Turbid, slimy sediment and pellicle	Turbid, slimy sediment and pellicle
Litmus milk	Alkaline with clearing	No change
Pigment production:		
Pyocyanin	—	—
Fluorescein	+	+
Fermentation:		
Glucose	+	+
Sucrose	+ (few —)	+ (few —)
Lactose	—	—
Reduction of Nitrates	+	+
Indol	—	—
H_2S	—	—

— = Negative: + = Positive.

603 cultures was characterized by either very feeble growth or no growth at 37°C., rather active liquefaction of gelatin, and peptonization of litmus milk. Group 2 containing 10 strains was found to grow well at 37°C. but did not liquefy gelatin or bring about any change in litmus milk. All lophotrichous forms were found to grow well at room temperature on nutrient agar slants. Growth was found to be filiform, abundant, and greyish-white. After an incubation period of a few days, a soluble yellow-green pigment penetrated the substrate, increasing in amount as aging progressed. In nutrient broth growth was initiated at the surface as delicate floccules which as they settled resulted in uniform turbidity. After incubation for several days a slightly viscid sediment accumulated. A yellow-green pigment was usually in evidence near the surface of the broth. As this pigment was colorless under reduced conditions, it was not apparent throughout the entire tube as long as the culture was actively metabolizing. On aging of the cultures, the pigment was observed to travel downward as oxygen

apparently diffused downward and oxidized the pigment to a colored state. The use of synthetic media showed that all lophotrichous strains produced fluorescein, but in no case could production of pyocyanin be demonstrated. Careful control of pH was found necessary in this study as fluorescein decolorizes in slightly acid solution and may be overlooked under such conditions.

Group 1 forms were found to liquefy gelatin with an initial crateriform type of liquefaction that slowly changed to the infundibuliform type. In most cases liquefaction was found to be pronounced at the end of three days at 18°C. Group 2 cultures showed no evidence of liquefaction in any trial at the end of 14 days.

Activity in litmus milk was found closely correlated with activity in gelatin. Group 1 was found to produce an alkaline reaction followed by clearing. In undisturbed tubes the alkaline reaction first appeared as a zone at the top and progressed downward. As the intensity of the daily zones was distinctly pronounced the medium took on a banded appearance. Clearing also began at the top of the tube and progressed downward. Reduction of litmus began at the bottom and after the fifth to seventh day was usually complete except for a shallow zone at the top of the tube. At the end of a week the milk usually became completely cleared and assumed a greenish-yellow color. Group 2 on the other hand did not produce a noticeable change in litmus milk.

The production of acid from glucose was observed in both groups of lophotrichous cultures. In nutrient broth, to which glucose was added, the medium turned slightly alkaline within one day but as incubation proceeded the medium slowly became acid, maximum acidity being reached in from three to five days. Following this the medium again became alkaline, and at the tenth day all signs of previous acidity had been obscured. The maximal acidity in glucose nutrient broth, although evident, was very slight, and was obscured if the original reaction of the substrate was slightly alkaline. Certain lophotrichous forms appeared to be able to attack sucrose. The acid from sucrose was found to appear more slowly than in the case of glucose but the acidity remained over a longer period of time. Lactose was not attacked by the members of either group.

All strains were found to be active denitrifiers. A few lophotrichous strains were found able to reduce the nitrogen of nitrates to the elemental form. Production of indol and hydrogen sulfide

were never observed with any of the 613 cultures. On potato slants all cultures grew luxuriently, producing a dirty green coloration.

These data indicate that the group of 603 cultures conformed to the description of *Bacterium fluorescens* (Flugge), Lehmann and Neumann *(17)*, although it includes isolations received from other laboratories as *Phytomonas tabaci, Ph. angulata, Ph. primulae, Ph. cerasi, Ph. vignae, and Ph. syringae,* as well as isolations received as *Pseudomonas fluorescens.* The other group of 10 cultures conformed to the description of *Bacterium putidum* (Flugge), Lehman and Neumann *(17)* and consisted entirely of organisms isolated from soil during the course of this investigation.

Serological studies.—With the discovery of the agglutination phenomena by Gruber and Durham *(10)* in 1896, a new means has been available for the identification of bacteria. Following this discovery, serological reactions on account of their great sensitivity and specificity have been employed to a large extent in assisting in disease diagnosis as well as in the species identification of bacteria. The union of bacterial cells with their respective antibodies (agglutinins) brings about a disappearance of the agglutinins from the serum in which they were present. Such a test known as agglutinin absorption as employed in this study, was first used clinically by Castellani *(3)* in 1902 and was established by Krumweide, Cooper, and Provost *(16)*. These authors state:

> "We have emphasized again and again the postulate that a completely positive reciprocal agglutinin absorption test is the ultimate criterion of serological likeness or identity of two bacterial cultures or strains and that absence of the completely positive reciprocal reaction is a definite criterion of unlikeness."

Although 603 of the 613 lophotrichous organisms were found to be similar to *Pseudomonas fluorescens* on the basis of morphology and biochemical reactions, this group included cultures previously identified as other species in the genus *Pseudomonas* as well as species in the genus *Phytomonas.* In a further effort to determine whether these 603 cultures formed a homogeneous group, serological reactions such as agglutination and agglutinin absorption were employed.

For the purpose of these serological studies 25 representative cultures including 6 recognized *Phytomonas* species, 8 cultures of *Pseudomonas fluorescens* and 1 culture of *Pseudomonas putida* were used. The source of these cultures is shown in table 3.

Table 3.—Source of cultures used in serological studies.

Culture No.	Source	Isolated by
BB	*Ph. tabaci* isolated from wildfire lesion, 1938	W. S. Beach, The Pennsylvania State College
11	*Ph. tabaci* isolated from wildfire lesion, 9/1937	W. S. Beach, The Pennsylvania State College
12	*Ph. tabaci* isolated from wildfire lesion, 2/1938	W. S. Beach, The Pennsylvania State College
527	*Ph. tabaci* isolated from wildfire lesion	E. E. Clayton, U. S. Department of Agriculture
16	*Ph. tabaci* isolated from wildfire lesion, 1923.	American Type Culture Collection
410	*Ph. angulata* isolated from angular leafspot, 1939	J. Johnson, University of Wisconsin
519	*Ph. primulae* isolated from leafspot of primrose	P. A. Ark, University of California
520	*Ph. cerasi* obtained from E. E. Wilson, University of California	W. H. Burkholder, Cornell University
524	*Ph. syringae* isolated from lima beans, 10/1937	W. H. Burkholder, Cornell University
204	*Ps. fluorescens* isolated from normal mature red clover	J. Naghski, The Pennsylvania State College
80	*Ps. fluorescens* isolated from normal tobacco, 1938	R. G. Harris, The Pennsylvania State College
521	*Ph. vignae* obtained from E. E. Wilson, University of California	W. H. Burkholder, Cornell University
353	*Ps. fluorescens* isolated from soil	J. Naghski, The Pennsylvania State College
504	*Ps. fluorescens* isolated from old red clover, 1939	J. Naghski, The Pennsylvania State College
630-1	*Ps. putida* isolated from compost heap, 1939	J. Naghski, The Pennsylvania State College
BBF	Single cell of *Ph. tabaci* BB isolated from wildfire	W. S. Beach, The Pennsylvania State College
$80F_2$	Single cell of *Ps. fluorescens* 80 isolated from normal tobacco, 1938	R. G. Harris, The Pennsylvania State College
204D	Single cell of *Ps. fluorescens* 204 isolated from normal mature red clover	J. Naghski, The Pennsylvania State College
519F	Single cell of *Ph. primulae* 519 isolated from leafspot of primrose	P. A. Ark, University of California
520A	Single cell of *Ph. cerasi* 510 obtained from E. E. Wilson, University of California.	W. H. Burkholder, Cornell University
521L	Single cell of *Ph. vignae* obtained from E. E. Wilson	W. H. Burkholder, Cornell University
524C	Single cell of *Ph. syringae* 524 isolated from lima beans, 10/1937	W. H. Burkholder, Cornell University
230	*Ps. fluorescens* isolated from normal young red clover, 1939	J. Naghski, The Pennsylvania State College
248	*Ps. fluorescens* isolated from normal young red clover, 1939	J. Naghski, The Pennsylvania State College
353	*Ps. fluorescens* isolated from soil	J. Naghski, The Pennsylvania State College

In a preliminary series of agglutination experiments two cultures in the Dawson "M" phase were used as antigens in animal inoculation for preparing agglutinating sera. These cultures were *Phytomonas tabaci* BB and *Pseudomonas fluorescens* 204. The agglutination reactions using the antisera so obtained with homologous and heterologous cultures are shown in table 4.

Table 4.—Agglutination reactions of "M" phase Phytomonas and Pseudomonas species with anti-sera prepared against a strain of Ph. tabaci and a strain of Ps. fluorescens in the "M" phase.

	PHYTOMONAS TABACI BB ANTI-SERUM DILUTIONS									PSEUDOMONAS FLUORESCENS 204 ANTI-SERUM DILUTIONS							
SPECIES OF BACTERIA	1-50	1-100	1-200	1-400	1-800	1-1600	1-3200	1-6400	1-12800*	1-50	1-100	1-200	1-400	1-800	1-1600	1-3200	1-6400	1-12800*
Ph. tabaci BB	4†	4	4	4	4	4	3	2	–	–	2	4	4	4	3	1	–	–
Ph. tabaci 11	4	4	4	2	–	–	–	–	·	2	4	2	1	–	–	–	–	–
Ph. tabaci 12	4	4	4	4	4	3	1	–	·	1	2	4	4	2	–	–	–	–
Ph. tabaci 527	4	4	4	4	4	4	4	2	–	–	2	4	4	4	3	2	–	–
Ph. tabaci 16	4	4	4	2	·	·	·	–	·	·	2	2	–	–	–	–	–	·
Ph. angulata 410	4	4	4	4	4	4	4	2	–	–	2	4	4	4	3	1	–	–
Ph. primulae 519	4	4	4	4	4	4	2	1	–	–	2	4	4	4	2	–	–	–
Ph. cerasi 520	4	4	4	4	4	4	4	2	–	2	4	4	4	4	4	2	–	–
Ph. vignae 521	4	4	4	2	·	–	·	–	–	2	4	4	4	2	–	–	–	–
Ph. syringae 524	4	4	4	4	2	–	–	–	–	2	4	4	4	3	1	–	–	–
Ps. fluorescens 204	–	2	4	4	4	4	2	1	–	4	4	4	4	4	4	4	2	–
Ps fluorescens 80	4	4	4	4	4	3	1	–	–	2	4	4	4	4	4	2	–	–
Ps. fluorescens 353	4	2	–	–	–	–	–	–	–	4	2	–	–	–	–	–	–	–
Ps. fluorescens 504	4	4	4	4	4	4	4	2	–	4	4	4	4	4	4	4	3	1
Ps putida 630-1	·	·	·	–	–	–	–	–	–	·	·	–	–		·	–	–	–

* Higher anti-serum dilutions and saline control were negative.
† Numbers refer to degree of macroscopic agglutination: 4 = complete; 1 = slight; · = no agglutination.

It will be noted in table 4 that all members of lophotrichous group 1 used in this experiment showed some antigenic relationship with each of the antisera, while *Ps. putida* 630-1 representing group 2 of the lophotrichous organism failed to show such antigenic relationship. It will also be noted that various strains of *Phytomonas tabaci* reacted in much lower dilution with the antiserum prepared with *Pseudomonas fluorescens* 204 than did *Phytomonas tabaci* BB. In this connection it is noteworthy that the two strains of *Phytomonas tabaci* showing the lowest titre against both antisera, strains 11 and 16 are the oldest isolations in the group and have been in laboratory cultivation for a much longer period of time than have any of the others. Strain 12 represents an isolation made more recently than 11 and 16 but not as recently as BB and strain number 527. The relationship between length of laboratory cultivation and degree of reaction with the two antisera is also ap-

parent with other species of *Phytomonas*. *Ph. vignae* 521 and *Ph. syringae* 524 are relatively old isolations and reacted poorly while, on the other hand, the other species of *Phytomonas* represented are more recent isolations and reacted to the titer of BB and 204. Antigenic differences in the case of *Pseudomonas fluorescens* strains, however, cannot be explained on this basis as all are relatively recent isolations.

In order to confirm the similarity in antigenic nature noted between *Phytomonas tabaci* BB and *Pseudomonas fluorescens* 204 as shown in table 4, an agglutinin absorption study was made using these organisms and their homologous antisera. The data obtained are presented in table 5.

Table 5.—Reciprocal agglutinin-absorption of "M" phase anti-sera and "M" phase antigens of Phytomonas tabaci BB and Pseudomonas fluorescens 204.

Serum	Absorbed by	Agglutinated with	Serum Dilutions								
			1-50	1-100	1-200*	1-400	1-800	1-1600	1-3200	1-6400	C
BB	BB	BB	-	-	-	-	-	-	-	-	-
		204	-	-	-	-	-	-	-	-	-
BB	204	BB	-	-	-	-	-	-	-	-	-
		204	-	-	-	-	-	-	-	-	-
204	BB	BB	-	-	-	-	-	-	-	-	-
		204	-	-	-	-	-	-	-	-	-
204	204	BB	-	-	-	-	-	-	-	-	-
		204	-	-	-	-	-	-	-	-	-

C = saline control.

The inability to demonstrate, table 5, either homologous or heterologous agglutination between *Phytomonas tabaci* BB and *Pseudomonas fluorescens* 204 after reciprocal absorption in titres as low as 1 to 50 when it was previously shown, table 4, that cross agglutination titres ranged from 1-800 to 1-3,200 indicates the very close antigenic relationship and similarity of these two microorganisms.

Serological studies employing single-celled cultures.—In order to make certain that the antigenic relationship found to exist among known strains of *Pseudomonas* and *Phytomonas* in these preliminary studies was not a result of impure cultures, 39 single celled isolations were made of 5 species of *Phytomonas* and 3 species of *Pseudomonas* by the method previously described. Only those single-cell strains were chosen for use as antigens which closely resembled the parent culture in all morphological, physiological, and serological characteristics, table 3. Representative single-cell strains in the Dawson "M" phase were used as antigens in animal inoculation and the agglutination reactions of these strains with the antisera obtained are presented in table 6.

Table 6.—Cross agglutination reactions of the "M" phase of Pseudomonas and Phytomonas species.

ANTIGENS	ANTISERA								
	BBF	$80F_2$	410	204D	519F	520A	521L	524C	412
BBF	2560†	5120	2560	2560	2560	5120	—	—	—
$80F_2$	2560	5120	2560	2560	5120	5120	—	—	—
410	2560	5120	2560	2560	5120	10240	—	—	—
204D	1280	5120	1280	2560	5120	5120	—	—	—
519F	1280	5120	1280	2560	5120	10240	—	—	—
520A	1280	5120	2560	2560	5120	10240	—	—	—
521L	320	640	160	1280	160	2560	10240	2560	—
524C	640	1280	160	80	80	40	5120	10240	—
412*	—	—	—	—	—	—	—	—	1280

* 412 is a culture of *Ps. aeruginosa* used for purposes of comparison.
† Numbers represent the reciprocal of the highest serum dilution showing macroscopic agglutination.

The data in table 6 shows that the strains of *Phytomonas tabaci* BBF, *Ph. angulata* 410, *Ph. primulae* 519F, *Ph. cerasi* 520A and *Pseudomonas fluorescens* 204D and $80F_2$ give cross agglutination reactions in almost the tame titre as obtained with the homologous organism. *Phytomonas vignae* 521L and *Ph. syringae* 524C, on the other hand, show considerably less antigenic similarity.

Agglutinin absorption tests employing the single-celled isolations were also used in a further effort to determine relationships within this group of cultures. The data obtained are presented in table 7.

Table 7.—Reciprocal agglutinin absorption of "M" anti-sera by "M" antigens of Pseudomonas and Phytomonas species.

SERUM	ABSORBED BY	ANTIGENS							
		BBF	$80F_2$	410	204D	519F	520A	521L	524C
	BBF	— 40*	— 40	— 40	+ 40	— 40	+ 40	— 40	— 40
	$80F_2$	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	410	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
BBF	204D	+ 640	+ 640	+ 160	— 40	— 40	— 40	— 40	— 40
	519F	+ 320	+ 320	— 40	— 40	— 40	— 40	— 40	— 40
	520A	+ 640	+ 640	+ 160	+ 40	— 40	— 40	— 40	— 40
	521L	+ 1280	+ 1280	+ 1280	+ 640	++ 640	++ 320	— 40	— 40
	524C	+ 1280	++ 1280	+ 1280	+ 640	+ 640	++ 160	— 40	— 40

* Numbers in this table are reciprocals of highest serum dilution showing any macroscopical agglutination. ++++, +++, ++, + denote degree of agglutination from complete to trace. Controls and higher dilutions were negative.

Table 7. (Continued)—Reciprocal agglutinin absorption of "M" anti-sera by "M" antigens of Pseudomonas and Phytomonas species.

Serum	Absorbed by	Antigens							
		BBF	$80F_2$	410	204D	519F	520A	521L	524C
	BBF	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	$80F_2$	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	410	— 40	— 40	— 40	++ 40	— 40	++ 40	— 40	— 40
	204D	+ 80	++ 320	+ 80	— 40	— 40	— 40	— 40	— 40
$80F_2$	519F	+ 640	++ 320	++ 160	— 40	— 40	— 40	— 40	— 40
	520A	+ 640	++ 320	+ 320	+ 40	— 40	— 40	++ 320	+ 160
	521L	++ 2560	+ 5120	++ 5120	++ 2560	+ 5120	+ 2560	— 40	— 40
	524C	+ 2560	++ 2560	+ 5120	+ 2560	+ 5120	++ 5120	— 40	— 40
	BBF	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	$80F_2$	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	410	— 40	— 40	— 40	++ 40	— 40	++ 40	— 40	— 40
	204D	+ 320	+ 320	+ 160	— 40	— 40	— 40	— 40	— 40
410	519F	++ 320	+ 160	— 40	— 40	— 40	— 40	— 40	— 40
	520A	+ 640	+ 640	+ 160	+ 40	— 40	— 40	— 40	— 40
	521L	+ 1280	++ 1280	++ 640	++ 640	+++ 640	— 40	— 40	— 40
	524C	++ 1280	+ 1280	+ 1280	+ 640	+++ 320	+ 1280	— 40	— 40
	BBF	+ 40	++ 40	+ 80	++ 160	++ 80	+ 160	+ 320	— 40
	$80F_2$	— 40	— 40	— 40	+ 160	— 40	+ 160	+ 320	— 40
	410	— 40	— 40	— 40	+ 640	— 40	+ 160	+ 320	— 40
	204D	+ 40	+ 40	— 40	— 40	— 40	— 40	+ 40	— 40
204D	519F	+ 80	— 40	— 40	+ 320	— 40	++ 80	++ 320	— 40
	520A	+ 160	++ 160	++ 40	++ 320	+ 40	— 40	+++ 320	+ 40
	521L	++ 1280	+++ 1280	+++ 1280	++ 1280	++ 1280	+ 1280	— 40	— 40
	524C	+ 1280	+ 1280	+++ 1280	+ 1280	+ 1280	+ 1280	— 40	— 40

Table 7. (Continued)—Reciprocal agglutinin absorption of "M" anti-sera by "M" antigens of Pseudomonas and Phytomonas species.

Serum	Absorbed by	Antigens							
		BBF	$80F_2$	410	204D	519F	520A	521L	524C
	BBF	+ 40	— 40	+ 40	+ 160	++ 80	+ 40	— 40	— 40
	$80F_2$	— 40	— 40	— 40	+++ 640	+++ 640	— 40	— 40	— 40
	410	— 40	— 40	— 40	+ 320	+++ 640	— 40	— 40	— 40
	204D	— 40	— 40	— 40	— 40	++ 320	— 40	— 40	— 40
519F	519F	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	520A	— 40	+ 40	+ 40	+ 160	++ 160	— 40	— 40	— 40
	521L	++ 1280	+ 2560	+++ 2560	+ 2560	+ 2560	+ 2560	— 40	— 40
	524C	+++ 1280	+++ 1280	++ 2560	++ 1280	++ 1280	++ 1280	— 40	— 40
	BBF	— 40	++ 80	++ 80	++ 320	+ 160	++ 320	+ 80	— 40
	$80F_2$	— 40	— 40	++ 80	+ 160	+ 80	++ 160	+ 320	— 40
	410	— 40	— 40	— 40	+ 40	— 40	++ 40	++ 80	— 40
	204D	— 40	— 40	— 40	— 40	— 40	+ 160	++ 160	— 40
520A	519F	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	520A	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	521L	+ 5120	+ 5120	+ 10240	+ 10240	+ 5120	++ 2560	— 40	— 40
	524C	++ 2560	+ 5120	+ 5120	+ 5120	++ 1280	++ 5120	+ 640	— 40
	BBF	— 40	— 40	— 40	+ 160	— 40	+ 80	+ 1280	++ 320
	$80F_2$	— 40	— 40	— 40	— 40	— 40	— 40	+++ 2560	+ 2560
	410	— 40	— 40	— 40	— 40	— 40	— 40	++ 2560	+ 1280
	204D	— 40	— 40	— 40	— 40	— 40	— 40	++ 5120	++ 5120
521L	519F	— 40	— 40	— 40	— 40	— 40	— 40	+ 2560	+ 2560
	520A	— 40	— 40	— 40	+ 320	+++ 40	— 40	+++ 1280	+ 1280
	521L	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	524C	— 40	— 40	— 40	+ 160	— 40	++ 40	++ 1280	— 40

Table 7. (Continued)—Reciprocal agglutinin absorption of "M" anti-sera by "M" antigens of Pseudomonas and Phytomonas species.

Serum	Absorbed by	Antigens							
		BBF	$80F_2$	410	204D	519F	520A	521L	524C
	BBF	— 40	— 40	— 40	— 40	— 40	+ 40	+ 640	+ 640
	$80F_2$	— 40	— 40	— 40	— 40	— 40	— 40	+ 640	+ 2560
	410	— 40	— 40	— 40	— 40	— 40	— 40	++ 640	+ 2560
	204D	— 40	— 40	— 40	— 40	— 40	— 40	— 40	++ 5120
524C	519F	— 40	— 40	— 40	— 40	— 40	— 40	+ 640	+ 2560
	520A	— 40	— 40	— 40	— 40	++ 80	— 40	++ 640	+ 2560
	521L	— 40	— 40	— 40	— 40	— 40	— 40	— 40	++ 640
	524C	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40

It will be noted in table 7 that reciprocal absorption (employing these cultures) indicates the antigenic identity of *Ph. tabaci* BBF, *Ph. angulata* 410, *Ph. primulae* 519F, *Ph. cerasi* 520A and *Pseudomonas fluorescens* $80F_2$ and 204D. *Ph. vignae* 521L and *Ph. syringae* 524C were found to reduce the titres of the other sera although not effecting complete removal of the agglutinins. The other cultures likewise did not completely remove the agglutinins from the *Ph. vignae* 521L and *Ph. syringae* 524C antisera, although effecting a reduction in titre. On the basis of these data, *Phytomonas syringae* 524C and *Ph. vignae* 521L appear to have a greater antigenic similarity to each other than either exhibits toward the remaining cultures. Thus in the "M" phase, they cannot be considered identical. It should be mentioned again, however, that the strains of *Phytomonas vignae* 521L and *Ph. syringae* 524C are the only cultures in this comparison which are not recent isolations. Previous work with *Phytomonas tabaci* strains which had been under laboratory cultivation for long periods of time indicated a progressive loss of antigenic similarity in the "M" phase as a result of laboratory cultivation, table 4. The data presented in tables 4 to 7 inclusive obtained from the study of organisms in the Dawson "M" phase indicates the very close antigenic relation of these cultures.

Antigenic relation of decapsulated green fluorescent bacteria.—The studies of Julianelle *(14)* have shown that antigenic relationships between cultures in the "M" phase may merely indicate that similar soluble specific polysaccharides are present in the capsular material of the cells and that upon decapsulation the same cells

show no antigenic similarity. For the purpose of determining whether the antigenic similarity found in this study was merely one of capsular material or actually involved the proteins of the cell, Dawson "S" forms of these strains of *Phytomonas* and *Pseudomonas* were obtained by growth of the organism in nutrient broth containing 10 per cent homologous immune serum. In the case of recent isolations included in this study little difficulty was encountered in obtaining the Dawson "S" phase. In the case, however, of the older isolates, *Phytomonas vignae* 521L and *Phytomonas syringae* 524C, considerable difficulty was encountered not only in obtaining the "S" phase but also in maintaining organisms in it. In these two cases the difficulty in maintaining the cells in the "S" phase was so great that the cultures finally used as antigens cannot be said with certainty to represent the "S" phase, as "M" variants appeared frequently in spite of all precautions.

Animals were immunized with the "S" phase strains and the antisera obtained were used in making agglutination tests with homologous and heterologous strains. The results are presented in table 8.

Table 8.—Cross agglutination reaction of the "S" phase of Pseudomonas and Phytomonas species.

ANTIGENS	ANTISERA								
	BBF	$80F_2$	410	204D	519F	520A	521L	524C	412
BBF	10240†	10240	10240	5120	5120	10240	5120	5120	—
$80F_2$	5120	10240	10240	5120	5120	10240	5120	5120	—
410	5120	10240	10240	5120	5120	5120	5120	5120	—
204D	5120	5120	10240	5120	10240	5120	5120	5120	—
519F	5120	10240	5120	5120	10240	10240	2560	5120	—
520A	5120	10240	10240	5120	10240	10240	5120	5120	—
521L	5120	5120	5120	10240	10240	10240	10240	5120	—
524C	5120	5120	10240	10240	5120	5120	5120	10240	—
412*	—	—	—	—	—	—	—	—	1240

* 412 is a culture of *Pseudomonas aeruginosa* used for the purpose of comparison.
† Numbers represent the reciprocal of the highest serum dilution showing macroscopic agglutination.

It will be noted in table 8 that this group of lophotrichous green fluorescent bacteria in the "S" phase showed definite antigenic similarity upon cross agglutination among all strains while culture No. 412 a monotrichic culture which was used as a control showed no antigenic relation with the eight lophotrichous "S" phase antisera. The relationship between *Phytomonas syringae* 524C and *Ph. vignae* 521L and the remaining members of the lophotrichous group appeared more pronounced than in any study conducted with "M" phase cells.

The first series of agglutinin absorption reactions made with organisms in the "S" phase and the antisera obtained from animal inoculation with "S" phase cells indicated that the absorbing ca-

pacity of "S" phase cells was less than those in the "M" phase. The titres obtained upon homologous and heterologous agglutination showed that a similar degree of absorption had occurred but seldom had the agglutinins been completely removed. For this reason three standard absorbing doses of antigen were used in later studies. Eight antisera produced with "S" phase cultures were each absorbed by the eight cultures in the "S" phase and then tested for the presence of agglutinins with homologous and heterologous cultures. The results are shown in table 9.

Table 9.—Reciprocal agglutinin absorption of "S" phase anti-serum and "S" phase antigens of Pseudomonas and Phytomonas species.

Serum	Absorbed by	Antigens							
		BBF	80F₂	410	204D	519F	520A	521L	524C
	BBF	++ 20*	+ 40	++ 20	+ 40	+ 80	++ 40	— 50	— 50
	80F₂	++ 80	+ 50	++ 20	+ 50	++ 50	+ 50	— 50	— 50
BBF	410	++ 20	+ 50	++ 20	+ 40	++ 50	++ 40	+ 50	— 50
	204D	+ 40	— 50	++ 20	+ 20	— 50	++ 20	— 50	— 50
	519F	+ 50	++ 50	+ 40	+ 50	++ 20	+ 50	— 50	— 50
	520A	— 50	— 50	— 50	— 50	— 50	— 50	— 50	— 50
	521L	++ 100	+ 100	+++ 50	+++ 50	+ 100	++ 100	++ 100	+ 100
	524C	+ 1600	+++ 1600	++ 1600	++ 1600	++ 1600	+++ 1600	— 50	— 50
	BBF	++ 40	+ 80	++ 40	+ 80	+ 40	+ 80	+ 50	++ 50
	80F₂	++ 50	++ 50	+ 50	— 50	++ 40	++ 50	+ 50	+ 50
	410	+ 80	++ 40	+ 40	— 50	— 50	++ 50	++ 50	++ 50
	204D	+ 40	++ 20	+ 80	+ 40	+ 40	++ 50	++ 50	+ 50
80F₂	519F	++ 50	+ 50	+ 50	+ 50	+ 50	— 50	— 50	++ 50
	520A	— 50	— 50	— 50	— 50	— 50	— 50	— 50	— 50
	521L	+ 100	+ 50	— 50	— 50	++ 50	++ 50	+ 50	+ 50
	524C	++ 1600	+++ 1600	+++ 1600	+++ 1600	++ 3200	++ 3200	— 50	— 50

* Numbers are reciprocals of the highest serum dilution showing macroscopic agglutination. ++++, +++, ++, + denote degree of agglutination from complete to trace. Higher dilutions and saline controls were negative.

Table 9. (Continued)—Reciprocal agglutinin absorption of "S" phase antiserum and "S" phase antigens of Pseudomonas and Phytomonas species.

SERUM	ABSORBED BY	ANTIGENS							
		BBF	$80F_2$	410	204D	519F	520A	521L	524C
410	BBF	+ 20	+ 40	+ 80	++ 20	++ 40	+ 40	— 50	— 50
	$80F_2$	++ 20	+ 20	++ 40	+ 40	+ 40	++ 20	— 50	— 50
	410	++ 20	++ 40	+ 80	+ 20	+ 80	+ 40	+ 50	+ 50
	204D	+ 20	+ 20	+ 20	+ 20	+ 40	++ 20	— 50	— 50
	519F	++ 50	++ 50	+ 50	— 50	— 50	++ 50	++ 50	+ 50
	520A	++ 50	++ 50	+ 100	++ 50	+ 50	+ 50	+ 100	+ 50
	521L	+ 100	+ 100	++ 50	+++ 50	+ 50	+ 100	+++ 50	++ 50
	524C	++ 3200	++ 3200	+++ 3200	++ 3200	+++ 3200	+++ 3200	— 50	— 50
204D	BBF	++ 40	+ 80	++ 40	+ 80	++ 40	+ 80	++ 50	++ 50
	$80F_2$	+ 20	+ 20	+ 20	+ 20	+ 50	+ 50	+ 50	+ 50
	410	+ 40	++ 40	++ 40	+ 40	+++ 50	++ 50	++ 50	++ 50
	204D	+ 20	+ 20	+ 20	+ 20	+ 50	+ 50	+ 50	++ 50
	519F	+++ 50	++ 50	+ 100	+ 100	++ 50	++ 50	++ 50	++ 50
	520A	+ 100	+ 50	+ 50	100	— 100	+++ 50	++ 50	++ 50
	521L	+++ 50	++ 50	+ 50	++ 50	+ 50	++ 50	++ 50	+ 50
	524C	+++ 1600	++ 800	++ 800	+++ 1600	++ 1600	++ 800	++ 50	— 50
519F	BBF	— 40	— 40	— 40	+ 80	+ 80	— 40	— 40	— 40
	$80F_2$	+++ 50	+ 50	+ 50	+ 50	+ 100	++ 50	++ 50	++ 50
	410	+ 80	++ 50	+ 50	+ 40	++ 50	++ 50	+ 50	+ 50
	204D	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	519F	+ 50	+ 50	++ 50	++ 50	++ 50	+ 50	— 50	+ 50
	520A	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	521L	+ 100	++ 50	++ 50	+ 100	+ 100	+ 100	+ 100	++ 50
	524C	++ 1600	+++ 800	+++ 800	++ 1600	++ 1600	++ 1600	— 50	— 50

Table 9. (Continued)—Reciprocal agglutinin absorption of "S" phase antiserum and "S" phase antigens of Pseudomonas and Phytomonas species.

Serum	Absorbed by	Antigens							
		BBF	$80F_2$	410	204D	519F	520A	521L	524C
520A	BBF	+ 80	— 40	++ 40	+ 80	— 40	++ 80	+ 40	+ 40
	$80F_2$	— 40	— 40	— 40	+ 80	— 40	+ 80	— 40	— 40
	410	++ 40	+ 40	— 40	+ 80	+ 40	+ 80	— 40	— 40
	204D	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	519F	++ 100	+ 100	+ 100	++ 50	++ 50	+ 100	++ 50	++ 50
	520A	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40
	521L	++ 100	++ 100	+ 100	+++ 50	+ 100	++ 100	+ 100	+++ 50
	524C	+++ 1600	+++ 1600	++ 1600	+++ 1600	++ 1600	+ 3200	— 50	— 50
521L	204D	++ 50	++ 50	+ 50	+ 50	++ 50	+++ 50	++ 400	++ 800
	519F	+++ 50	++ 50	++ 50	++ 50	++ 50	+ 100	+ 100	+ 200
	520A	— 40	— 40	— 40	— 40	— 40	— 40	+ 800	++ 400
	521L	— 50	— 50	— 50	— 50	— 50	— 50	— 50	— 50
	524C	— 50	— 50	— 50	— 50	— 50	— 50	— 50	— 50
524C	204D	++ 50	++ 50	+ 50	+ 50	++ 50	++ 50	++ 800	++ 800
	519F	++ 50	+ 50	— 50	— 50	— 50	— 50	+ 100	+ 200
	520A	+++ 50	+ 50	— 50	++ 50	++ 50	— 50	++ 100	++ 400
	521L	— 50	— 50	— 50	— 50	— 50	— 50	— 50	— 50
	524C	— 50	— 50	— 50	— 50	— 50	— 50	— 50	— 50

Table 9 shows the complete reciprocal removal of agglutinins in all antisera except that prepared against *Phytomonas vignae* 521L and *Ph. syringae* 524C. The results obtained by three absorptions with *Phytomonas cerasi* 520A, *Ph. tabaci* BBF, *Ph. angulata* 410, *Ph. primulae* 519F, are comparable to those obtained by three absorptions with the strains of *Pseudomonas fluorescens* 204D and $80F_2$.

The results presented in table 9 indicate that the protein fractions of *Phytomonas tabaci* BBF, *Ph. angulata* 410, *Ph. primulae* 519F, *Ph. cerasi* 520A and *Pseudomonas fluorescens* 204D and $80F_2$

are antigenically identical. The data cannot be said to indicate such antigenic identity with respect to *Phytomonas syringae* 524C and *Ph. vignae,* but it likewise cannot be said with certainty in the case of these two strains that all cells used in the production of antisera and as antigens in agglutination reactions were in the "S" state. Failure of reciprocal agglutinin absorption to indicate identity in these cases had rather been expected because of the tendency of variant "M" forms to appear in any cultivation of these strains apart from homologous antisera. That this accounts for the failure to demonstrate equivalent reciprocal agglutinin absorption in tests of these strains with the other members of the group receives additional confirmation in a study of the absorptive capacity of *Phytomonas vignae* 521L. In what approximated the "S" phase in the case of this species all agglutinins were removed from each of the other sera, however, the other organisms with the exception of *Phytomonas syringae* 514C failed to remove agglutinins from *Phytomonas vignae* 521L antiserum to the equivalent extent. This can only be accounted for on the assumption that the so-called "S" phase of *Phytomonas vignae* 521L contained all of the antigens possessed by *Phytomonas tabaci* BBF, *Ph. angulata* 410, *Ph. cerasi* 520A, *Ph. primulae* 519F and *Pseudomonas fluorescens* 204D and $80F_2$ in the "S" phase but, in addition, some other antigenic fraction was present such as could be accounted for by the presence of capsular material due to the "M" variants which always appeared in the cultivation of the organism.

Variation in antigenicity of green fluorescent bacteria.—The foregoing experiments on a number of green fluorescent bacteria have indicated that a close relationship exists between the bacterial pathogen *Ph. tabaci* and the soil saprophyte *Ps. fluorescens.* The concept that one and the same organism may at one time be parasitic and another time saprophytic is not at all unusual in the light of our knowledge of bacterial variation. Studies dealing with bacterial variation have shown *(11)* that numerous bacterial pathogens undergo temporary variations as a result of change in environment. Such adaptations frequently include correlated variations in which the colony morphology, virulence, and antigenic characters of the cells vary. In this study as shown on the following pages similar alterations in antigenic structure with a correlated variation with respect to virulence have been noted.

It is not feasible to discuss the subject of bacterial variation in this paper. Reference has been made to an extensive review *(11).* Certain workers *(2)* question the value of serological reactions as a means of measuring relationship between plant pathogens while others *(22)* believe a definite positive correlation exists. The

authors realize the many pitfalls confronting workers in this field of study but believe that the earlier conclusions drawn from investigations which did not take into consideration dissociation phenomena or separate the different bacterial phases for study are not valid objections at the present time. Recent reports *(20)* stating the lack of relation between pathogenicity and agglutination phenomena have apparently erred in a similar manner in disregarding the phase study of the cultures employed.

The authors consider that serological techniques measure the sum of the morphological and physiological characteristics (including pathogenicity) of the germ plasm of bacterial cells. Such techniques are a measure of the chemical composition of cells or the chemical composition of the surface areas of bacterial cells. It is well established that numerous groups of organisms in the "M" phase have slightly different morphological, physiological, and serological characteristics. However, if the surface covering of polysaccharide material is removed from these different groups, the remaining "S" cells are identical. The polysaccharide surface covering of such "S" cells is known to be a product of the cell environment. Pathogenicity is just as much a variable factor as other physiological and morphological characteristics of microorganisms. In many diseases of man and other animals such as typhoid fever, pneumonia, "Bangs" disease, pullorum etc., serological analysis is employed as the final and especially sensitive test indicating the presence of the bacteria responsible for the disease. So proved and accepted is this method of serological techniques that in no case is it required upon obtaining a positive serological result to inject a second person with a typhoid or other disease organism and produce the disease to be certain that the first person in reality had typhoid fever. It is well known that many pathogens maintained for any prolonged period on laboratory culture media become avirulent. In some instances loss of virulence occurs slowly and at other times fairly rapidly. It is also known that virulence may be enhanced when the microorganism is in a suitable environment or host. It is known that in epidemics of disease the virulence of the microorganism varies from one epidemic to another. All gradations of virulence from a practically complete avirulent form to a form of maximum virulence may exist at different times. Wolfe and Foster in giving the name wildfire to this leafspot disease of tobacco may have inadvertently described how rapidly normal soil and plant forms may become pathogenic under suitable conditions. Since pathogenicity is a relative term and must be considered in relation to host susceptibility; bacterial variation of plant pathogens should of necessity

take into consideration the physiology and environment of the host plant.

It should not be assumed that since *Phytomonas tabaci* and *Ps. fluorescens* in the "S" form are identical that they are at all times equally pathogenic. The pathogenicity of a microorganism as stated above is determined equally by the environment or host of the bacterial cell and by the inherent nature of the organism itself. The authors are therefore presenting this new concept that *Ph. tabaci* and *Ps. fluorescens* are identical inherently and that the harmless, relatively avirulent organism *Ps. fluorescens* under suitable conditions can readily increase in virulence until it possesses all the characteristics of the pathogen known as *Ph. tabaci*. *Pseudomonas fluorescens* therefore is considered a potential hazard in that under suitable environmental conditions, this form builds a characteristic polysaccharide material which makes it similar in all respects to *Ph. tabaci*.

The poor titres obtained with cells of *Pseudomonas fluorescens* 353, table 4, in agglutination tests using antisera prepared against *Pseudomonas fluorescens* 204 and *Phytomonas tabaci* BB led to a further investigation of this strain. This culture, 353, was used as an antigen in animal inoculation and the antiserum obtained was tested against cells of *Pseudomonas fluorescens* 204 and found to react well, 1 to 6400, although cells of 353 agglutinated only in dilutions of 1 to 50 and 1 to 100 in antiserum prepared against 204. Two attempts were made to change the antigenic structure of 353. One transfer of 353 in nutrient broth containing 10 per cent of homologous immune serum resulted in cells which reacted with 204 antiserum in a dilution of 1 to 6400. Five serial transfers in tobacco extract broth resulted in cells of strain 353 which reacted with 204 antiserum in a dilution of 1 to 3200. It would appear that some slight capsular difference had served to mask the antigenic similarity of 353 to other members of the group. Table 10 shows this in tabular form.

That similar slight antigenic changes constantly occur in nature is suggested by a study of members of *Pseudomonas fluorescens* isolated at frequent intervals from growing clover representing young to mature plants. Agglutination reactions of a few of these isolates showing the general trend as the plant approaches maturity are presented in table 11. Culture 353 was isolated from the fertile soil early in the spring. Culture 230 was isolated from the epiphytic flora of a very young clover plant. Culture 248 was isolated from a half grown clover plant and cultures 204 and 504 were isolated from mature plants.

Table 10.—Effect of various environmental conditions on the agglutinability of Pseudomonas fluorescens.

	Ps. fluorescens 353 Anti-serum Dilutions									Ps. fluorescens 204 Anti-serum Dilutions								
Bacteria	1-50*	1-100	1-200	1-400	1-800	1-1600	1-3200	1-6400	1-12800*	1-50	1-100	1-200	1-400	1-800	1-1600	1-3200	1-6400	1-12800
Ps. fluorescens 204	4†	4	4	4	4	2	–	–	–	4	4	4	4	4	4	4	4	2
Ps. fluorescens 353	4	4	4	4	4	4	4	2	–	4	2	–	–	–	–	–	–	–
Ps. fluorescens 353 AS‡	4	4	4	4	4	4	4	3	1	4	4	4	4	4	4	4	3	1
Ps. fluorescens 353 TE§	4	4	4	4	4	4	2	–	–	4	4	4	4	4	4	4	2	–

* Serum dilutions—higher dilutions and saline controls were negative.
† Numbers refer to degree of macroscopic agglutination. 4 = complete; 1 = slight; – = negative.
‡ 353 AS = after growth in homologous anti-serum.
§ 353 TE = after growth in unheated tobacco extracts.

Table 11 shows that the antiserum prepared against *Phytomonas tabaci* BB either failed to react or at best reacted only to a slight degree with isolates obtained early in the life of the plant. Week by week antigentic similarity became more pronounced until at the end of the season the typical member of *Pseudomonas fluorescens* isolated from the mature plant proved antigenically identical with *Phytomonas tabaci* BB in the "M" phase. This suggests that the capsular antigenicity of *Phytomonas tabaci* is a reflection of continued reproduction as an epiphyte.

Table 11.—Agglutination reactions of progressive isolations from clover of Pseudomonas fluorescens with anti-serum of Phytomonas tabaci BB.

	Serum Dilutions							
Bacteria	1-50	1-100	1-200	1-400	1-800	1-1600	1-3200	1-6400*
Ps. fluorescens 353	4†	2	–	–	–	–	–	–
Ps. fluorescens 230	–	–	–	–	–	–	–	–
Ps. fluorescens 248	4	4	4	3	1	–	–	–
Ps. fluorescens 204	–	2	4	4	4	4	2	
Ps. fluorescens 504	4	4	4	4	4	4	4	2

* Higher dilutions and saline control were negative.
† Numbers refer to degree of macroscopic agglutination, 4 = complete, 1 = slight, – = negative.

DISCUSSION AND APPLICATION OF RESULTS

Practical value of the antigenic identity found to exist between Ph. tabaci and Ps. fluorescens.— In view of the results previously obtained showing the close similarity and identity of *Ph. tabaci* and *Ps. fluorescens* it was realized that a serological method was available for determining the presence of leafspot organisms in soils and on normal leaves as well as on diseased plants. The agglutination technique described under the section on methods and as employed in the earlier part of this bulletin was used to detect the presence of leafspot bacteria in seedbed soils and on normal plants. Such a method can be readily employed in detecting the presence of leafspot organisms in seedbed soils before and after steaming, in the field and on tobacco and other plants which may not be diseased.

Influence of steaming on microorganisms in tobacco seedbeds.— Although the steaming of tobacco seedbeds has been recommended by almost every writer on the subject as a means of eliminating wildfire and angular leafspot organisms from the soil, there has been found in the literature no record of any serious study of the effects of soil steaming upon these organisms. The authors began an investigation of this phase of the subject in the spring of 1937 in cooperation with the late Otto Olson of the United States Department of Agriculture and have continued this study through 1938, 1939, and 1940. In the course of the investigation a large number of soil samples and seedlings from Lancaster county seedbeds were collected and examined for bacteria by the methods previously described.

Table 12 shows the numbers of bacteria, fungi, and green fluorescent organisms obtained per gram of soil and the presence of wildfire microorganisms in 20 representative soils samples collected in April and May, 1940, from seedbeds before steaming.

The same seedbeds after steaming in accordance with the sanitary recommendations made for the control of tobacco wildfire were again examined. Not more than four beds represent the work of one operator. The results of the bacteriological analyses of the same 20 seedbeds shown in table 12 after steaming are shown in table 13.

It is shown in table 13 that the wildfire organism was found present in all 20 seedbed soils following the steam treatment. This indicates that the steaming of seedbed soils as practiced in the district does not result in sterilization as the common terminology, "seedbed sterilization", has led many to believe. It does not result in the destruction of certain of the less resistant bacteria and con-

Table 12.—Numbers of microorganisms in Lancaster county seedbed soils before steam treatment.

FARM NUMBER	ORGANISMS PER GRAM OF SOIL			
	Bacteria	Fungi	Green fluorescent bacteria	Wildfire organisms
1	11,000,000	45,000	100	Present
2	34,000,000	90,000	200	Present
3	12,000,000	47,000	50	Present
4	9,500,000	5,000	100	Present
5	8,500,000	22,000	10	Present
6	7,600,000	35,000	100	Present
7	17,000,000	41,000	50	Present
8	19,000,000	10,000	200	Present
9	24,000,000	85,000	100	Present
10	65,000,000	180,000	200	Present
11	22,000,000	90,000	100	Present
12	38,000,000	75,000	100	Present
13	18,000,000	34,000	100	Present
14	29,000,000	37,000	200	Present
15	16,000,000	21,000	50	Present
16	13,000,000	9,500	10	Present
17	27,000,000	23,000	100	Present
18	31,000,000	32,000	100	Present
19	19,000,000	16,000	50	Present
20	8,100,000	13,000	10	Present

sequently can not be compared to the pasteurization process used in dairy and food industries. The data supplied in table 13 supports the observations of Chapman and Anderson *(4)* who noted that seedbed steaming had either little or no relation to the occurrence of wildfire in the field. Attention is directed to the fact that the data presented in table 13 represent in some cases analyses made immediately after soil steaming, while in other cases the samples were taken several days following steaming. In the latter instances some recontamination of the bed may have occurred prior to sampling, but in the case of beds on farms 1, 2, 10, 11, and 15 any significant recontamination prior to sampling appears unlikely.

The examination of more than 200 seedbed soils failed to reveal a single case in which steaming resulted in the absence of green fluorescent bacteria. It will be noted that in those cases where sampling was done immediately following steaming, a reduction in the number of these organisms was found to have taken place during the steam treatment. This reduction, however, was not great enough in most cases to suggest that complete destruction could be accomplished by any reasonable increase in the duration of the treatment. This conclusion was substantiated in an experi-

Table 13.— Numbers of microorganisms in Lancaster county seedbed soils following steam treatment; analysis of the same seedbeds as were reported in table 12.

Farm Number	Interval Between Steaming and Sampling	Organisms per Gram of Soil			
		Bacteria	Fungi	Green fluorescent bacteria	Wildfire organisms
1	1 hour	350,000	60	10	Present
2	2 hours	1,400,000	175	100	Present
3	1 week	1,500,000	450	200	Present
4	2 weeks	1,200,000	200	200	Present
5	3 weeks	2,100,000	5,000	200	Present
6	3 weeks	1,800,000	40,000	100	Present
7	3 weeks	3,900,000	5,000	100	Present
8	3 weeks	2,400,000	200	100	Present
9	1 week	3,100,000	450	200	Present
10	1 day	2,800,000	300	100	Present
11	4 hours	650,000	70	50	Present
12	2 weeks	9,500,000	30,000	200	Present
13	3 weeks	19,000,000	70,000	200	Present
14	2 days	2,600,000	100	50	Present
15	2 hours	970,000	40	10	Present
16	4 days	3,200,000	800	10	Present
17	2 weeks	11,000,000	9,000	200	Present
18	2 days	1,700,000	200	10	Present
19	3 weeks	9,400,000	6,500	100	Present
20	2 weeks	3,500,000	5,900	100	Present

mental test. Steaming for a period of 1 hour failed to destroy all green fluorescent organisms in the upper 4 inches of soil.

In any consideration of the effects of steaming it should be borne in mind that it is much more difficult to effect destruction of microorganisms in a heavy clayey soil than in a light sandy soil. Consequently the average Lancaster county grower could not expect the destructive effect which may be obtained on the lighter soils of certain other tobacco districts. Even the reaction of the soil of Lancaster seedbeds is unfavorable from the standpoint of disinfection. Acid soils are more easily sterilized than are natural soils and very few seedbeds have been found in the Lancaster area with a pH value below 6.8.

Increase in bacterial numbers following steaming.—Table 13 shows that in those cases in which the second samples were taken one to three weeks after the steam treatment, there appears to have been an actual increase in the number of green fluorescent bacteria in the seedbed soils. In order to determine whether such increases in the number of microorganisms was a characteristic change following steaming, the seedbeds of six Lancaster county farms were examined over a 7-week interval of time for bacteria,

fungi, and the presence of green fluorescent forms. From 10 to 30 isolations of green fluorescent bacteria were made from each seedbed sample, and by means of the agglutination test, using antisera prepared with known strains of the wildfire organism, the percentage of this type of green fluorescent bacteria was determined. The data are presented in table 14.

Table 14.—Results of interval examination of six typical seedbeds in Lancaster county.

FARM NUMBER	SAMPLING DATE	ORGANISMS PER GRAM OF SOIL			
		Bacteria	Fungi	Green fluorescent bacteria	Wildfire organisms*
		number	*number*	*number*	*per cent*
1	April 17	1,800,000	40,000	100	17
	May 16	138,000,000	95,000	200	33
	June 4	670,000,000	80,000	200	70
2	April 17	3,900,000	5,000	100	10
	May 16	110,000,000	70,000	200	67
	June 4	980,000,000	40,000	200	82
3	April 17	3,000,000	450	50	20
	May 16	110,000,000	30,000	100	17
	June 4	1,200,000,000	400,000	200	45
4	April 17	19,000,000	25,000	100	40
	May 16	105,000,000	40,000	100	55
	June 4	430,000,000	3,000	200	75
5	April 17	2,400,000	200	10	15
	May 16	115,000,000	4,000	50	10
	June 4	900,000,000	30,000	100	30
6	April 17	650,000	60	10	50
	May 16	240,000,000	55,000	200	17
	June 4	870,000,000	38,000	100	60

* Wildfire organisms present expressed as percentages of isolations of green-fluorescent bacteria found identical with the wildfire organism on the basis of serological tests.

It is to be noted in table 14 that during the period of seven weeks intervening between the first and third samplings, there was a considerable increase in both bacteria and fungi. Among the types of bacteria taking part in this reproduction were the green fluorescent organisms. Of more importance, however, the data indicates an increase in that part of the green fluorescent flora that is associated with the bacterial leafspots of tobacco. These data show that not only are there more green fluorescent bacteria in the typical steamed seedbed some seven weeks after seeding than are present at the time of seeding but that the percentage of these organisms which can be identified as wildfire or angular leafspot bacteria actually increases during this period. These data may serve to explain the observations of Clinton and McCormick *(6)* that steamed beds had a greater amount of wildfire than unsteamed

beds. Inasmuch as all of these beds contained seedling tobacco, the possibility exists that this reproduction may have taken place on the plants rather than in the soil and that the organisms were washed into the soil in the watering of the beds. Other experimental evidence, however, indicates that at least a part of this increase in numbers of leafspot organisms actually took place in the soil as a substrate. Well-manured soil was placed in pots and steamed for 20 minutes. Following this heat treatment the wildfire organism was added and counts were made at intervals over a period of three weeks, using the same methods employed previously. No crop was grown in this experiment but the results showed that an actual increase in numbers of the wildfire organism had occurred during the three weeks period, although many spore-formers and other microorganisms were present as competitors.

It is worthy of note in connection with the data presented in table 14 that bordeaux mixture was used at intervals on all six beds.

It may be concluded therefore that the mere presence of the leafspot organism in seedbed soils has not been the determining factor leading to wildfire and that some other factor or factors have been responsible for the occurrence of leafspot diseases of tobacco.

Bacterial flora of the tobacco plant.—During the course of this study the bacterial flora of hundreds of tobacco samples from both the seedbed and the field was determined. Data collected in 1937 did not include serological analyses of the green fluorescent bacteria encountered on the plant. Beginning in 1938, however, the green fluorescent isolates obtained from such plant material were subjected to agglutination studies and the percentages of leafspot organisms were determined. A summary of the data obtained showing the incidence of the types of microorganisms encountered is shown in table 15.

It is to be noted in table 15 that the three gram-positive types encountered appear to compose a very stable portion of the bacterial flora of the leaf, while the gram-negative types, on the other hand, were found to vary considerably. The leafspot organisms formed a portion of the normal flora of almost all tobacco plants.

It was found that the percentages of leafspot organisms in the green fluorescent flora may vary from 0 to 100 per gram in the case of very young plants, but that during the course of the growing season the percentages rise steadily until practically all green fluorescent organisms on a plant in the latter part of the season may be wildfire and angular leafspot in nature. This increase was found to be directly associated with the extent of the reproduction

Table 15.—Bacterial flora of Pennsylvania tobacco in seedbed and field.

TYPE OF BACTERIA	STABILITY OF POPULATION	NUMBERS USUALLY FOUND PER GRAM
Cocci, gram-positive, both chromogenic and non-chromogenic	Stable	Less than 10,000
Rods, gram-positive, non-spore-forming	Stable	Less than 10,000
Rods, gram-positive, spore-forming	Stable	Less than 75,000
Rods, gram-negative, non chromogenic	Variable	From less than 1,000 to more than 1,000,000
Rods gram-negative, pink pigment	Variable	From very few to several million
Rods, gram-negative, yellow pigment	Variable	From a few thousand to a few million
Rods, gram-negative, green-fluorescent pigment	Variable	From very few to 20,000,000

of the green fluorescent bacteria on the plant. In other words, the greater the number of generations of green fluorescent bacteria upon the plant, the greater is the percentage of forms which can be identified as leafspot organisms. Upon the aerial portions of these plants, the leafspot organisms reproduce in accordance with climatic conditions and host nutrition, and only under conditions of lowered host resistance does the association become detrimental to the green plant. At such times the green fluorescent forms fluctuate in number. The conditions which were associated with this fluctuation are shown in table 16.

Table 16 shows that if the relative humidity has been low, as is often the case in the summer, the bacterial population of tobacco leaves will usually be found to be less than 100,000 per gram, with the stable gram-positive portion of the flora comprising the greater part of the count. Under such conditions it is often necessary to utilize enrichment cultivation on selective media to demonstrate the presence of the gram-negative types. On the other hand, high relative humidity in seedbed or field is followed by a rapid rise in the gram-negative flora of the leaf. The particular type or types of gram-negative bacteria which reproduce most abundantly under such conditions appears to depend upon the nutrition and physiology of the tobacco plant. Under satisfactory conditions of nutrition and environment, saccharolytic yellow rods play the major role in this gram-negative development, while under conditions of high nitrogen nutrition, proteolytic yellow rods

Table 16.—Factors associated with the relative numbers of the several types of organisms which comprise the normal flora of the growing tobacco plant.

TYPE OF BACTERIA	GENERAL REMARKS	CONDITIONS ASSOCIATED WITH DOMINANCE
Gram-positive organisms, rods and cocci	Dominant type in cases of counts below 100,000 per gram. Presence demonstrated by desiccation of sample in cases of counts much above 100,000.	The characteristic flora of tobacco following periods of low humidity.
Gram negative rods, non-chromogenic	Includes representatives of *Proteus*, *Aerobacter*, and *Achromobacter*. A few can be demonstrated on most samples. Rarely dominant on Pennsylvania tobacco.	The characteristic flora of a certain type of tobacco not grown in Pennsylvania. Found on some Pennsylvania tobacco following periods of high humidity.
Gram-negative rods, pink pigment	Can usually be demonstrated on Pennsylvania tobacco but rarely in large numbers.	Characteristic of certain inferior tobaccos of the flue-cured type.
Gram-negative yellow pigment (a) Saccharolytic type	These peritrichic rods can be demonstrated on any tobacco of good quality and when dominant the counts range from 100,000 to 1,000,000.	This is the typical flora of good tobacco of most types following periods of moderate to high humidity.
(b) Proteolytic type	These monotrichic rods can be demonstrated on most tobacco. When dominant the counts range from 500,000 to 3,000,000	This is characteristic of certain tobacco with unbalanced nutrition following periods of high humidity.
Gram-negative rods, green fluorescent pigment	These lophotrichic rods can be found on almost all samples of growing tobacco. When dominant the counts range from 500,000 to 20,000,000. High counts often associated with high counts of proteolytic yellow rods and non-chromogenic gram-negative rods.	This is the typical flora of tobacco with high nitrogen and low potassium uptake following periods of moderate to high humidity.

and green fluorescent bacteria reproduce so rapidly as to obscure the other forms. A violent upset of the microbial equilibrium in the direction of either proteolytic yellow or green fluorescent forms was found by the authors to be indicative of unfavorable environmental conditions in the seedbed or the field.

Among the agricultural plants subjected to study by the authors in their efforts to establish the nature of the normal bacterial flora of living plants, red clover received considerable attention. From the first it was apparent that the flora of the normal red clover plant closely resembles the flora of the normal tobacco plant. The same stable population of gram-positive organisms was found present; the same saccharolytic yellow rods flourished in periods of moderate to high humidity. More important from the standpoint of this research, green fluorescent rods were found to be a normal part of the flora of the clover plant and were found to appear in great numbers under conditions similar to those inciting such reproduction on the tobacco plant. Most important of all, however, it was found that this green fluorescent flora of mature clover plants could be identified as the organisms of wildfire and angular leafspot, while on the other hand the green fluorescent flora of very young clover proved serologically different from known wildfire and angular leafspot bacteria. In other words the antigenic nature of isolates from young clover was found to be quite different from that of the leafspot organisms, while the antigenic nature of isolates from old clover proved identical to that of the leafspot organisms.

In order to determine whether the microorganisms of leafspot diseases could survive as free living forms in the soil, their ability to utilize various simple substrates to meet their nutritional requirements was examined. Four known cultures associated with wildfire and angular leafspot, 1. *Ph tabaci* No. 591[1]; 2. *Ph. tabaci* No. 12; 3. *Ph. tabaci* No. 532; and 4. *Ph. angulatum* No. 410, were inoculated into seven simple culture media. The ability of these organisms to grow upon repeated subculture in these media is shown in table 17.

Table 17 shows that all four leafspot organisms were able to grow on these seven substrates with the exception of the nitrated coal medium. Very few disease causing organisms possess the ability to grow on such simple substrates. This fact emphasizes the unusual ability of the green fluorescent bacteria associated with leafspot disturbances to grow readily in an environment less suitable than is commonly encountered in the soil. These data also indicate that leafspot organisms could readily live from year to year in soil even in the absence of tobacco refuse.

The evidence presented indicates that the organisms of wildfire and angular leafspot are widely distributed in nature and occur normally on many plants as harmless saprophytes.

[1] The authors wish to express their thanks to W. S. Beach for cultures No. 591 and No. 12; E. E. Clayton for culture No. 532 and J. Johnson for No. 410.

Table 17.—Growth of wildfire and angular leaf spot organisms on various substrates.

SUBSTRATE	ORGANISM			
	Wildfire No. 591	Wildfire No. 12	Wildfire No. 532	Angular Leaf spot No. 410
Glucose-asparagin mineral salts	Excellent	Excellent	Excellent	Excellent
Glucose-ammonium sulfate-mineral salts	Good	Fair	Good	Good
Asparagin-mineral salts	Excellent	Good	Excellent	Good
Malic acid-ammonium sulfate-mineral salts	Good	Good	Good	Good
Succinic acid- ammonium sulfate-mineral salts	Good	Fair	Good	Fair
Crude petroleum-ammonium sulfate-mineral salts	Fair	Poor	Fair	Fair
Nitrated Coal solution-ammonium sulfate-mineral salts	Fair	None	Poor	Poor

ANTIGENIC RELATIONSHIPS

The results of these investigations have demonstrated that the leafspot organism is always present in soil and that *Phytomonas tabaci* is identical morphologically, culturally, and serologically with the common soil form *Pseudomonas fluorescens*. This is a new concept in that it was formerly held that *Phytomonas tabaci* was a parasitic form which did not live independently of its host and survived over winter in tobacco sheds on old tobacco, etc. The complete reciprocal agglutinin absorption which was demonstrated in both the Dawson "M" and "S" phases between culture BBF a virulent culture of *Phytomonas tabaci* and *Pseudomonas fluorescens* 204D a common soil bacterium leaves little if any doubt, that the two forms are identical. The value of serological analyses in showing species relations as well as the alteration occurring in virulence and antigenic characters in bacterial variation is well established.

The question naturally arises as to how a normal soil bacterium such as *Pseudomonas fluorescens* will suddenly become involved in a disease which spreads so rapidly that the name, wildfire accurately describes it. The question may be answered by a consideration of the ecological relation existing between the tobacco plant and the bacterium. In nature it is characteristic that an approximately balanced condition exists between contending species. Such a balance is apparent in healthy tobacco where leafspot organisms are present on the leaf in fairly large numbers, as well as being present in the soil. Any factor which disturbs this balance could lead to an altered relationship between the plant and its

normal bacterial flora. Such disturbances with resulting leafspot troubles were recognized and described long before the serious disease outbreaks of 1917 *(13 and 15)*. The relationship of such disturbances to leafspot diseases were emphasized by investigators of the 1917 epidemics *(8 and 23)*. Similar findings of the authors of this paper will appear in subsequent publications.

Although these studies indicate that the peculiar antigenic components which characterized the "M" phase of *Phytomonas tabaci* are enhanced by reproduction of the organism as a plant epiphyte, these studies do not indicate that such progressive changes in the nature of the organism lead to a degree of virulence capable of overcoming the resistance of a normal plant. Strain 80 used in this experimental work was isolated from a normal, healthy, tobacco plant which showed no evidence of disease at any time during the season although other plants grown under less favorable conditions in nearby plots were seriously damaged. Strain 80, it may be added, was found to be more virulent on a susceptible host than any strain procured from other investigators.

These studies have shown not only the very close relationship that exists between *Phytomonas tabaci* and *Pseudomonas fluorescens* but also that there is little if any distinction between other plant pathogens such as *Ph. primulae, Ph. cerasi, Ph. vignae, Ph. syringae*, and *Pseudomonas fluorescens.* The demonstration of the relationship between these numerous forms indicates the fallacy of considering and naming an organism a distinct species because of its ability to attack different hosts. Such a procedure is not followed with tuberculosis bacteria. Cattle, hogs, man, and fowl are all susceptible to tuberculosis but the organisms causing the disease in these four different animal groups are recognized as being practically identical.

SUMMARY

In a study of a large number of green fluorescent gram-negative bacteria isolated from seedbed soils as well as plant material from seedbed and field which were compared with known cultures, the authors feel that the following conclusions are warranted at this time from the data obtained:

1. An extensive serological study employing single-cell isolations indicates that the organism associated with the leafspot disease known as wildfire is identical antigenically with the common saprophyte, *Pseudomonas fluorescens.*
2. From serological studies it is concluded that *Phytomonas angulata, Ph. primulae* and *Ph. cerasi* are also identical with the saprophyte *Pseudomonas fluorescens.* Due to the in-

ability to maintain cultures of *Phytomonas vignae* and *Ph. syringae* in the "S" phase, it has not been possible to show the identity of these with *Pseudomonas fluorescens*, but the data obtained suggest the possibility of such identity.

3. A study of the transformation of the capsular antigenicity of *Pseudomonas fluorescens* on normal clover plants permits the conclusion that the antigenic specificity of the wildfire organism in the "M" phase is a reflection of capsular changes occurring during the reproduction of the organism as a harmless epiphyte.
4. Seedbed steaming as practiced in Lancaster county does not result in the elimination of the bacteria associated with the leafspot diseases of tobacco. On the contrary these organisms are more prevalent following steaming than immediately prior thereto.
5. Tobacco in the Lancaster area is produced as a rule in the presence of leafspot organisms, inasmuch as the organisms associated with leafspot diseases have been found in all seedbeds examined and on most plant material from seedbed and field.
6. Inasmuch as the organisms have been found on most normal tobacco as well as on diseased plants, the appearance of the disease can not be attributed to the mere presence of the organisms but to some other factor or factors.

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